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(54) DNA fragment encoding a polypeptide having nitrile hydratase activity, a transformant containing the DNA fragment and a process for the production of amides using the transformant.

(57) The present invention discloses the amino acid sequence and nucleotide sequence of the α- and β-subunits of two types of nitrile hydratase derived from Rhodococcus rhodochrous J-I. The DNA fragment encoding nitrile hydratase is inserted into an expression vector and the recombinant vector is used for transformation. The transformant contains multiple copies of the gene and can produce much higher levels of nitrile hydratase than conventionally used microorganisms.

EP 0 445 646 A2

The present invention relates to a DNA fragment derived from Rhodococcus rhodochrous J-I and encoding a polypeptide having nitrile hydratase activity which hydrates nitriles to amides. The invention also relates to a recombinant DNA containing the above DNA fragment, and a transformant transformed with the recombinant DNA. The present invention further relates to a method of producing nitrile hydratase using the transformant and of amides using nitrile hydratase.

Nitrile hydratase or nitrilase is known as an enzyme that hydrates nitriles to amides. Microorganisms that produce nitrile hydratase include those belonging to the genus Bacillus, the genus Bacteridium, the genus Micrococcus and the genus Brevibacterium (See, JP-B-62-21517/1989, USP No. 4,001,081), the genus Corynebacterium and the genus Nocardia (See, JP-B-56-17918/1981, USP No. 4,248,968), the genus Pseudomonas (See, JP-B-59-37951/1984, USP No. 4,637,982), the genus Rhodococcus, the genus Arthrobacter and the genus Microbacterium (See, JP-A-61-162193/1986, EP-A-0188316), and Rhodococcus rhodochrous (See, JP-A-2-470/1990, EP-A-0307926).

Nitrile hydratase has been used to hydrate nitriles to amides. In the invention, microorganisms are engineered to contain multiple copies of a recombinant DNA encoding nitrile hydratase according to a recombinant DNA technology. The recombinant produces a remarkably high level of nitrile hydratase compared with conventionally used microorganisms.

The present inventors previously disclosed a DNA fragment derived from Rhodococcus sp. N-774 (FERM BP-1936) which also encodes a polypeptide having nitrile hydratase activity (JP-A-2-119778/1988).

In contrast, the present inventors utilizes a DNA fragment derived from Rhodococcus rhodochrous J-I for the production of nitrile hydratase. We isolated the gene encoding nitrile hydratase, inserted the gene into a suitable plasmid vector and transformed an appropriate host with the recombinant plasmid, thus successfully obtained the transformant producing nitrile hydratase which has high activity also on aromatic nitriles.

The present invention relates to

(1) a $DNA^{(H)}$ fragment encoding a polypeptide having nitrile hydratase activity, said polypeptide comprising the $\alpha^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 1 and the $\beta^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 2;

(2) a $DNA^{(L)}$ fragment encoding a polypeptide having nitrile hydratase activity, said polypeptide comprising the $\alpha^{(L)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 3 and the $\beta^{(L)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 4;

(3) the $DNA^{(H)}$ fragment of (1) which contains a nucleotide sequence encoding said $\alpha^{(H)}$- and $\beta^{(H)}$-subunits, comprising the DNA sequence of the $\alpha^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 5 and the DNA sequence of the $\beta^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 6;

(4) the $DNA^{(L)}$ fragment of (2) which contains a nucleotide sequence encoding said $\alpha^{(L)}$- and $\beta^{(L)}$-subunits, comprising the DNA sequence of the $\alpha^{(L)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 7 and the DNA sequence of the $\beta^{(L)}$subunit as defined in the Sequence Listing by SEQ ID: No. 8;

(5) a recombinant DNA comprising the $DNA^{(H)}$ or the $DNA^{(L)}$ of (1)-(4) in a vector;

(6) a transformant transformed with the recombinant DNA of (5);

(7) a method for the production of nitrile hydratase which comprises culturing the transformant as described in (6) and recovering nitrile hydratase from the culture;

(8) a method for the production of amides which comprises hydrating nitriles using nitrile hydratase as described in (7) to form amides; and

(9) a method for the production of amides which comprises culturing the transformant as described in (6), and hydrating nitriles using the resultant culture, isolated bacterial cells, treated matter thereof, or a fixed material of them, to form amides.

The present invention is described in detail as follows.

The present invention is carried out by the steps (1)-(8):

(1) Isolation and Purification of Nitrile Hydratase and Partial Amino Acid Sequencing of Nitrile Hydratase

Two types of nitrile hydratase (designated as H type and L type, respectively) are isolated and purified from Rhodococcus rhodochrous J-I (FERM BP-1478) and the both enzymes are separated into α and β subunits using HPLC. N-Terminal amino acid sequence each of the subunits is determined and shown in the Sequence Listing by SEQ ID: Nos. 9-12.

(2) Preparation of a DNA Probe for a Nitrile Hydratase Gene

A DNA probe is prepared from JM105/pYUK121 (FERM BP-1937) as described in JP-A-2-119778/1990 due to the high degree of homology in the amino acid sequence between the nitrile hydratase β subunit of Rhodococcus sp. N-774 described in said Japanese Patent Official Gazette and those of Rhodococcus rhodochrous J-I. Plasmid pYUK121 containing nitrile hydratase gene derived from Rhodococcus sp. N-774 is prepared from a JM105/pYUK121 culture. pYUK121 DNA is digested with SphI and SalI. The SphI-SalI fragment contains the nitrile hydratase gene (shown in the Sequence Listing by SEQ ID: No. 13) of Rhodococcus sp. N-774. The DNA fragment is radiolabeled.

(3) Detection of a DNA Segment Containing a Nitrile Hydratase Gene from the Chromosome ofRhodococcus rhodochrousJ-I

Chromosomal DNA is prepared from a Rhodococcus rhodochrous J-I culture. The chromosomal DNA is digested with restriction enzymes and hybridized to the probe described in (2) using the Southern hybridization method [Southern, E.M., J. Mol. Biol. 98, 503 (1975)].

Two DNA fragments of a different length are screened.

(4) Construction of a Recombinant Plasmid

A recombinant plasmid is constructed by inserting the chromosomal DNA fragment as prepared in (3) into a plasmid vector.

(5) Transformation and Screening for a Transformant Containing the Recombinant Plasmid

Transformants are prepared using the recombinant plasmid as described in (4). The transformant containing the recombinant plasmid is selected using the probe as described in (2) according to the colony hybridization method [R. Bruce Wallace et. al., Nuc. Aci. Res. 9, 879 (1981)]. Additionally, the presence of the nitrile hydratase gene in the recombinant plasmid is confirmed using the Southern hybridization method. The plasmids thus selected are designated as pNHJ10H and pNHJ20L.

(6) Isolation and Purification of Plasmid DNA and Construction of the Restriction Map

Plasmid DNAs of pNHJ10H and pNHJ20L as prepared in (5) are isolated and purified. The restriction map of the DNAs is constructed (Fig. 1) to determine the region containing nitrile hydratase gene.

(7) DNA Sequencing

The extra segment of the inserted DNA fragment in pNHJ10H and pNHJ20L is excised using an appropriate restriction enzyme. The inserted DNA fragment is then used for sequencing. The nucleotide sequence of the DNA fragment (SEQ: ID Nos. 14, 15) reveals that it contains the sequence deduced from the amino acid sequence as described in (1).

(8) Production of Nitrile Hydratase Using the Transformant and Conversion of Nitriles to Amides

The transformant as described in (8) is cultured. The bacterial cells are mixed with nitriles, a substrate of nitrile hydratase, and amides are produced.

Rhodococcus rhodochrous J-I was deposited with Fermentation Research Institute, Agency of Industrial Science and Technology, and was assigned the accession number FERM BP-1478. A transformant TGI/pNHJ10H containing pNHJ10H as described in (5) and a transformant TGI/pNHJ20L containing pNHJ20L as described in (5) were deposited with the above and assigned the accession number FERM BP-2777 and FERM BP-2778, respectively.

Any vectors including a plasmid vector (e.g., pAT153, pMP9, pHC624, pKC7, etc.), a phage vector (e.g., λgtII (Toyobo), Charon 4A (Amersham), etc.) may be used. Enzymes which may be used include SphI, SalI, EcoRI, BamHI, SacI, and the like, which are commercially available (Takara Shuzo). Various hosts may be used for transformation including but not limited to E. coli JM105 and E. coli TGI.

Culture media for the transformant are those ordinarily used in the art.

Conversion of nitriles to amides is carried out using nitrile hydratase, crude nitrile hydratase, the culture of the transformant, the isolated bacterial cells or treated matter thereof, and the like, prepared from the culture of the transformant.

Suitable nitriles in the invention include aromatic nitriles having 4-10 carbon atoms in the aromatic moiety and aliphatic nitriles having 2-6 carbon atoms, which are described in the European Patent Publication No. 0,307,926. Typical examples of the nitriles are 4-, 3- and 2-cyanopyridines, benzonitrile, 2,6-difluorobenzonitrile, 2-thiophene carbonitrile, 2-furonitrile, cyanopyrazine, acrylonitrile, methacrylonitrile, crotonitrile, acetonitrile and 3-hydroxypropionitrile.

The present invention discloses the amino acid sequence and nucleotide sequence of the α- and β-subunits of two types of nitrile hydratase derived from Rhodococcus rhodochrous J-I. The DNA fragment encoding nitrile hydratase is inserted into an expression vector and the recombinant vector is used for transformation. The transformant contains multiple copies of the gene and can produce much higher levels of nitrile hydratase than conventionally used microorganisms.

Fig. 1 shows restriction maps of recombinant plasmids, pNHJ10H and pNHJ20L.

The present invention is illustrated by the following Example.

The following abbreviations are used in the Example.

TE:	Tris-HCl (10 mM; pH 7.8), EDTA (1 mM, pH 8.0)
TNE:	Tris-HCl (50 mM; pH 8.0), EDTA (1 mM, pH 8.0), NaCl (50 mM)
STE:	Tris-HCl (50 mM; pk 8.0), EDTA (5 mM, pH 8.0), Sucrose (35 mM)
2×YT medium:	1.6% Trypton; 1.0% Yeast extract, 0.5% NaCl

Example

(1) Isolation and Purification of Nitrile Hydratase and Partial Amino Acid Sequencing of Nitrile Hydratase

Rhodococcus rhodochrous J-I was cultured in a medium (3 g/ℓ of yeast extract, 0.5 g/ℓ of KH_2PO_4, 0.5 g/ℓ of K_2HPO_4, 0.5 g/ℓ of $MgSO_4 \cdot 4H_2O$, 0.01 g/ℓ of $CoCl_2$, and 3 g/ℓ of crotonamide, pH 7.2) at 28° C for 80 hours. The bacterial cells were harvested. 50 g of the bacterial cells was disrupted and fractionated with ammonium sulfate. The sample was dialyzed and the dialysate was centrifuged. The supernatant was loaded on DEAE-Cellulofine chromatography, Phenyl-Sepharose chromatography, Sephadex G-150 chromatography and Octyl-Sepharose chromatography. Two fractions with enzyme activity were obtained and dialyzed. The dialysates were loaded on a high performance liquid chromatography using a reversed phase column (Senshu Pak VP-304-1251, Senshu Kagaku), and two respective subunits (α and β) were obtained. N-terminal amino acid sequence of $\alpha_1^{(H)}$-, $\beta_1^{(H)}$-, $\alpha_1^{(L)}$- and $\beta_1^{(L)}$-subunits was determined using an Applied Biosystems model 470A protein sequencer. The amino acid sequences are shown in the Sequence Listing by SEQ ID: Nos. 9-12.

(2) Preparation of a DNA Probe for Nitrile Hydratase Gene

E. coli JM105 (FERM BP-1937) containing pYUK121 was cultured in 100 ml of 2×YT medium containing 50 μg/ml of ampicillin at 30° C overnight (12 hours). The bacterial cells were harvested and TNE was added to the cells. The cell suspension was then centrifuged. 8 ml of STE and 10 mg of lysozyme were added to the pellet. The mixture was incubated at 0° C for five minutes followed by the addition of 4 ml of 0.25M EDTA. 2 ml of 10% SDS and 5 ml of 5M NaCl were then added to the mixture at room temperature. The resultant mixture was incubated at 0-4° C for three hours and then ultracentrifuged. 1/2 volume of 30% PEG 6000 was added to the supernatant. The mixture was incubated at 0-4° C overnight (12 hours) and centrifuged. TNE was added to the pellet to bring the volume to 7.5 ml and CsCl was then added to the suspension. The mixture was centrifuged to remove proteins. Then, 300-500 mg/ml of ethidium bromide was added to the supernatant. The mixture was transferred to a centrifuge tube. The tube was heat-sealed and then ultracentrifuged. cccDNA was extracted using a peristaltic pump. A bit more than equal amount of isopropyl alcohol saturated with water was added to the extract to rid of ethidium bromide. The sample was dialyzed against TE. About 3 ml of purified pYUK121 was obtained.

pYUK121 DNA was digested with SphI and SalI, resulting in a 2.07 kb DNA fragment containing a nitrile hydratase gene derived from Rhodococcus sp. N-774. The fragment was radiolabeled with ^{32}P to produce a probe. The nucleotide sequence of the probe is shown in the Sequence Listing by SEQ ID: No. 13.

(3) Preparation of a DNA Fragment Containing a Nitrile Hydratase Gene of Chromosome

Rhodococcus rhodochrous J-I was cultured in 100 ml of a medium (10 g/ℓ of glucose, 0.5 g/ℓ of KH_2PO_4, 0.5 g/ℓ of K_2HPO_4, 0.5 g/ℓ of $MgSO_4 \cdot 7H_2O$, 1 g/ℓ of yeast extract, 7.5 g/ℓ of peptone, 0.01 g/ℓ of $CoCl_2$, 7.5 g/ℓ of urea, 1% glycine or 0.2 μg/ml of ampicillin, 1 ℓ of water, pH 7.2). The bacterial cells

were harvested and the pellet was washed with TNE. The pellet was then suspended in 10 ml of TE. 4 ml of 0.25M EDTA, 10-20 mg of lysozyme, 10-20 mg of achromoprotease and 10 ml of 10×SDS were added to the suspension. The suspension was incubated at 37° C for three hours. 15 ml of phenol was added to the suspension. The mixture was incubated at room temperature for 15 minutes and then centrifuged. The upper layer was removed, and 0.7 ml of 2.5M sodium acetate and diethyl ether were added to the supernatant. The mixture was centrifuged and the upper layer was discarded. Two volumes of ethanol were added to the bottom layer and DNA was removed with a glass rod. DNA was rinsed for five minutes each with TE:ethanol 2:8, 1:9, and 0:10 (v/v). DNA was then resuspended in 2-4 ml of TE (37° C). 10 μl of a mixture of RNase A and T_1 was added to the suspension and the mixture was incubated at 37° C. An equal amount of phenol was added to the mixture which was then centrifuged. More than equal amount of ether was added to the supernatant. The mixture was centrifuged again, and the upper layer was discarded and the bottom layer was saved. The bottom layer was dialyzed against 2 l of TE containing a small amount of chloroform overnight and further dialyzed against fresh TE for 3-4 hours. 4 ml of crude chromosomal DNA was obtained.

10 μl of TE, 3 μl of reaction buffer (10×) and 2 μl of SacI were added to 15 μl of crude chromosomal DNA. The mixture was incubated at 37° C for an hour and electrophoresed on an agarose gel at 60 V for three hours. The Southern hybridization of chromosomal DNA was carried out using the probe as described in (2). About 6.0 kb and 9.4 kb fragments were found to show a strong hybridization.

15 μl of chromosomal DNA was digested with SacI and electrophoresed on an agarose gel, as described above. 6.0 kb and 9.4 kb DNA fragments were cut out from the gel and taken in three volumes each of 8M $NaClO_4$. After solubilization, each solution was dotted on GF/C (Whatman) filter paper (6 mm in diameter). Ten drops (≃ 100 μl) of TE containing 6M $NaClO_4$ and then ten drops (≃ 100 μl) of 95% ethanol were added to the filter paper. The paper was air-dried for 3 minutes and placed in 0.5 ml Eppendorf tube. 40 μl of TE was added to the tube and the whole was incubated at 47° C for 30 minutes. The tube was then centrifuged. About 40 μl of the supernatant was obtained which contained 6.0 kb and 9.4 kb DNA fragments containing a nitrile hydratase gene of chromosomal DNA.

The method of inserting the 6.0 kb DNA fragment into a vector is described below. The same method is applied for the insertion of the 9.4 kb DNA fragment into a vector.

(4) Insertion of the Chromosomal DNA Fragment into a Vector

10 μl of TE, 3 μl of reaction buffer (10×) and 2 μl of SacI was added to 10 μl of pUC19. The mixture was incubated at 30° C for an hour. 2 μl of 0.25M EDTA was added to the mixture to stop the reaction. Then, 7 μl of 1m Tris-HCl (pH 9) and 3 μl of BAP (bacterial alkaline phosphatase) were added to the mixture. The mixture was incubated at 65° C for an hour. TE was then added to the mixture to make a total volume to 100 μl. The mixture was extracted 3× with an equal amount of phenol. An equal amount of ether was added to the extract. The bottom layer was removed and 10 μl of 3M sodium acetate and 250 μl of ethanol were added to the bottom layer. The mixture was incubated at -80° C for 30 minutes, centrifuged, dried, and resuspended in TE.

5 μl of pUC19 DNA thus obtained and 40 μl of the 6.0 kb DNA fragment as described in (3) were mixed. 6 μl of ligation buffer, 6 μl of ATP (6 mg/ml) and 3 μl of T4 DNA ligase were added to the mixture. The mixture was incubated at 4° C overnight (12 hours) to produce the recombinant plasmid containing the 6.0 kb DNA fragment encoding the desired enzyme in the SacI site of pUC19.

(5) Transformation and Screening of Transformants

E. coli TGI (Amersham) was inoculated into 10 ml of 2×YT medium and incubated at 37° C for 12 hours. After incubation, the resultant culture was added to fresh 2×YT medium to a concentration of 1%, and the mixture was incubated at 37° C for two hours. The culture was centrifuged and the pellet was suspended in 5 ml of cold 50 mM $CaCl_2$. The suspension was placed on ice for 40 minutes and then centrifuged. 0.25 ml of cold 50 mM $CaCl_2$ and 60 μl of the recombinant DNA as described in (4) were added to the pellet. The mixture was incubated at 0° C for 40 minutes, heat-shocked at 42° C for two minutes, placed on ice for five minutes, and added to 10 ml of 2×YT medium. The mixture was incubated at 37° C for 90 minutes with shaking, then centrifuged. The pellet was suspended in 1 ml of 2×YT medium, and two 10 μl aliquots of the suspension were plated on a 2×YT agar plate containing 50 μg/ml of ampicillin separately. The plate was incubated at 37° C. The colony grown on the plate was selected by the colony hybridization method: The colony was transferred to a nitrocellulose filter and digested. The DNA was fixed on the filter and hybridized to the probe as described in (2). The filter was autoradiographed and

a recombinant colony was selected. Additionally, the presence of a nitrile hydratase gene in the transformant was confirmed according to the Southern hybridization method.

(6) Isolation and Purification of Recombinant Plasmid and Construction of the Restriction Map of the Inserted DNA Fragments

The transformant selected as described in (5) was grown in 100 ml of 2×YT medium containing 50 μg/ml of ampicillin at 37° C overnight (12 hours). The bacterial cells were harvested and TNE was added to the cells. The cells were collected again by centrifugation, and 8 ml of STE and 10 mg of lysozyme were added to the cells. The mixture was incubated at 0° C for five minutes. 4 ml of 0.25M EDTA, 2 ml of 10% SDS (at room temperature) and 5 ml of 5M NaCl were added to the mixture. The mixture was incubated at 0-4° C for three hours, and ultracentrifuged. 1/2 volume of 30% PEG 6000 was added to the supernatant. The mixture was incubated at 0-4° C overnight (12 hours) and centrifuged again. TNE was added to the pellet to bring the volume up to 7.5 ml. CsCl was added to the suspension to rid of proteins. Then, 300-500 mg/ml of ethidium bromide was added to the supernatant and the mixture was transferred to a centrifuge tube. The tube was heat-sealed and ultracentrifuged. cccDNA was removed using a peristaltic pump. A bit more than equal amount of isopropyl alcohol saturated with water was added to cccDNA to remove ethidium bromide. The DNA sample was dialyzed against TE, resulting in about 3 ml of purified recombinant DNA. The recombinant plasmid thus obtained containing a 6.7 kb DNA fragment was designated as pNHJ10H (The recombinant plasmid containing a 9.4 kb DNA fragment was designated as pNHJ20L).

These plasmid DNAs were digested with EcoRI, BamHI, PstI, SacI and SalI. The restriction maps were constructed and are shown in Fig. 1.

(7) DNA Sequencing

The location of a nitrile hydratase gene in the DNA fragment of pNHJ10H was determined according to the restriction map constructed and to the Southern hybridization method. An extra segment in pNHJ10H was cleaved off with PstI and SalI: The 6.0 kb DNA fragment resulted in 1.97 kb. Similarly, an extra segment in pNHJ20L was cleaved off with EcoRI and SacI: The 9.4 kb DNA fragment resulted in 1.73 kb.

These DNA fragments were sequenced by the Sanger method [Sanger, F., Science 214: 1205-1210 (1981)] using M13 phage vector. The nucleotide sequence of the 1.97 kb DNA fragment (pNHJ10H) and the 1.73 kb DNA fragment (pNHJ20L) are shown in the Sequence Listing by SEQ ID: No. 14 and SEQ ID: No. 15, respectively.

The amino acid sequence deduced from the nucleotide sequence was found fully identical to the amino acid sequence as determined in (1). The sequence analysis also revealed that the DNA fragment contained the sequence coding for the α- and β-subunits.

(8) Production of Nitrile Hydratase Using the Transformant and Conversion of Nitriles to Amides Using Nitrile Hydratase

TG1 /pNHJ10H and TG1/pNHJ20L were inoculated into 10 ml of 2×YT medium containing 50 μg/ml of ampicillin and incubated at 30° C overnight (12 hours). 1 ml of the resultant culture was added to 100 ml of 2×YT medium (50 μg/ml of ampicillin, 0.1 g of $CoCl_2 \cdot 6H_2O$/ℓ). The mixture was incubated at 30° C for 4 hours. IPTG was added to the mixture to a final concentration of 1 mM. The mixture was incubated at 30° C for 10 hours. After harvesting the cells, the cells were suspended in 5 ml of 0.1 M phosphate buffer (pH 7.5). The suspensions were disrupted by sonification for 5 min and centrifuged at 12,000 ×g for 30 min. The resulting supernatants were used for the enzyme assay. The enzyme assay was carried out in a reaction mixture (12 ml) containing 50 mM potassium phosphate buffer (pH 7.5), 6 mM benzonitrile and an appropriate amount of the enzyme. The reaction was carried out at 20° C for 30 min and stopped by the addition of 0.2 ml 1 M HCl. The amount of benzamide formed in the reaction mixture was determined by HPLC. As a control, the mixture obtained by the same procedure as described above but from E. coli TG1 was used. The levels of nitrile hydratase activity in cell-free extracts of E. coli containing pNHJ10H and pNHJ20L were 1.75×10^{-3} and 6.99×10^{-3} units/mg, respectively, when cultured in 2×YT medium in the presence of $CoCl_2$ and IPTG. Benzamide was found in the reaction mixture of TG1/pNHJ10H and pNHJ20L, whereas no benzamide was found in the reaction mixture of TG1 .

(1) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 203 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\alpha^{(H)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

```
                5                  10                  15
MetSerGluHisValAsnLysTyrThrGluTyrGluAlaArgThr
            20                  25                  30
LysAlaIleGluThrLeuLeuTyrGluArgGlyLeuIleThrPro
        35                  40                  45
AlaAlaValAspArgValValSerTyrTyrGluAsnGluIleGly
    50                  55                  60
ProMetGlyGlyAlaLysValValAlaLysSerTrpValAspPro
65                  70                  75
GluTyrArgLysTrpLeuGluGluAspAlaThrAlaAlaMetAla
80                  85                  90
SerLeuGlyTyrAlaGlyGluGlnAlaHisGlnIleSerAlaVal
            95                  100                 105
PheAsnAspSerGlnThrHisHisValValValCysThrLeuCys
        110                 115                 120
SerCysTyrProTrpProValLeuGlyLeuProProAlaTrpTyr
    125                 130                 135
LysSerMetGluTyrArgSerArgValValAlaAspProArgGly
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                    140                 145                 150
ValLeuLysArgAspPheGlyPheAspIleProAspGluValGlu
                155                 160                 165
ValArgValTrpAspSerSerSerGluIleArgTyrIleValIle
            170                 175                 180
ProGluArgProAlaGlyThrAspGlyTrpSerGluGluGluLeu
        185                 190                 195
ThrLysLeuValSerArgAspSerMetIleGlyValSerAsnAla
    200
LeuThrProGlnGluValIleVal
```

(2) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 229 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\beta^{(H)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

```
                5                   10                  15
MetAspGlyIleHisAspThrGlyGlyMetThrGlyTyrGlyPro
            20                  25                  30
ValProTyrGlnLysAspGluProPhePheHisTyrGluTrpGlu
        35                  40                  45
GlyArgThrLeuSerIleLeuThrTrpMetHisLeuLysGlyIle
    50                  55                  60
SerTrpTrpAspLysSerArgPhePheArgGluSerMetGlyAsn
65                  70                  75
GluAsnTyrValAsnGluIleArgAsnSerTyrTyrThrHisTrp
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                                    80                  85                  90
LeuSerAlaAlaGluArgIleLeuValAlaAspLysIleIleThr
                                95                  100                 105
GluGluGluArgLysHisArgValGlnGluIleLeuGluGlyArg
                            110                 115                 120
TyrThrAspArgLysProSerArgLysPheAspProAlaGlnIle
                        125                 130                 135
GluLysAlaIleGluArgLeuHisGluProHisSerLeuAlaLeu
                    140                 145                 150
ProGlyAlaGluProSerPheSerLeuGlyAspLysIleLysVal
                155                 160                 165
LysSerMetAsnProLeuGlyHisThrArgCysProLysTyrVal
            170                 175                 180
ArgAsnLysIleGlyGluIleValAlaTyrHisGlyCysGlnIle
        185                 190                 195
TyrProGluSerSerSerAlaGlyLeuGlyAspAspProArgPro
    200                 205                 210
LeuTyrThrValAlaPheSerAlaGlnGluLeuTrpGlyAspAsp
215                 220                 225
GlyAsnGlyLysAspValValCysValAspLeuTrpGluProTyr
LeuIleSerAla
```

(3) INFORMATION FOR SEQ ID NO: 3

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 207 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\alpha^{(L)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

```
                5                  10                  15
MetThrAlaHisAsnProValGlnGlyThrLeuProArgSerAsn
            20                  25                  30
GluGluIleAlaAlaArgValLysAlaMetGluAlaIleLeuVal
        35                  40                  45
AspLysGlyLeuIleSerThrAspAlaIleAspHisMetSerSer
    50                  55                  60
ValTyrGluAsnGluValGlyProGlnLeuGlyAlaLysIleVal
65                  70                  75
AlaArgAlaTrpValAspProGluPheLysGlnArgLeuLeuThr
            80                  85                  90
AspAlaThrSerAlaCysArgGluMetGlyValGlyGlyMetGln
        95                  100                 105
GlyGluGluMetValValLeuGluAsnThrGlyThrValHisAsn
    110                 115                 120
MetValValCysThrLeuCysSerCysTyrProTrpProValLeu
125                 130                 135
GlyLeuProProAsnTrpTyrLysTyrProAlaTyrArgAlaArg
            140                 145                 150
AlaValArgAspProArgGlyValLeuAlaGluPheGlyTyrThr
        155                 160                 165
ProAspProAspValGluIleArgIleTrpAspSerSerAlaGlu
    170                 175                 180
LeuArgTyrTrpValLeuProGlnArgProAlaGlyThrGluAsn
185                 190                 195
PheThrGluGluGlnLeuAlaAspLeuValThrArgAspSerLeu
            200                 205
IleGlyValSerValProThrThrProSerLysAla
```

(4) INFORMATION FOR SEQ ID NO: 4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 226 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\beta^{(L)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

```
                    5                  10                  15
MetAspGlyIleHisAspLeuGlyGlyArgAlaGlyLeuGlyPro
            20                  25                  30
IleLysProGluSerAspGluProValPheHisSerAspTrpGlu
        35                  40                  45
ArgSerValLeuThrMetPheProAlaMetAlaLeuAlaGlyAla
    50                  55                  60
PheAsnLeuAspGlnPheArgGlyAlaMetGluGlnIleProPro
65                  70                  75
HisAspTyrLeuThrSerGlnTyrTyrGluHisTrpMetHisAla
                80                  85                  90
MetIleHisHisGlyIleGluAlaGlyIlePheAspSerAspGlu
            95                  100                 105
LeuAspArgArgThrGlnTyrTyrMetAspHisProAspAspThr
        110                 115                 120
ThrProThrArgGlnAspProGlnLeuValGluThrIleSerGln
    125                 130                 135
LeuIleThrHisGlyAlaAspTyrArgArgProThrAspThrGlu
140                 145                 150
AlaAlaPheAlaValGlyAspLysValIleValArgSerAspAla
            155                 160                 165
SerProAsnThrHisThrArgArgAlaGlyTyrValArgGlyArg
        170                 175                 180
ValGlyGluValValAlaThrHisGlyAlaTyrValPheProAsp
    185                 190                 195
ThrAsnAlaLeuGlyAlaGlyGluSerProGluHisLeuTyrThr
200                 205                 210
ValArgPheSerAlaThrGluLeuTrpGlyGluProAlaAlaPro
            215                 220                 225
AsnValValAsnHisIleAspValPheGluProTyrLeuLeuPro
Ala
```

(5) INFORMATION FOR SEQ ID NO: 5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 609 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\alpha^{(H)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

```
                15                  30                  45
GTGAGCGAGCACGTCAATAAGTACACGGAGTACGAGGCACGTACC
        60                  75                  90
AAGGCGATCGAAACCTTGCTGTACGAGCGAGGGCTCATCACGCCC
       105                 120                 135
GCCGCGGTCGACCGAGTCGTTTCGTACTACGAGAACGAGATCGGC
       150                 165                 180
CCGATGGGCGGTGCCAAGGTCGTGGCCAAGTCCTGGGTGGACCCT
       195                 210                 225
GAGTACCGCAAGTGGCTCGAAGAGGACGCGACGGCCGCGATGGCG
       240                 255                 270
TCATTGGGCTATGCCGGTGAGCAGGCACACCAAATTTCGGCGGTC
       285                 300                 315
TTCAACGACTCCCAAACGCATCACGTGGTGGTGTGCACTCTGTGT
       330                 345                 360
TCGTGCTATCCGTGGCCGGTGCTTGGTCTCCCGCCCGCCTGGTAC
       375                 390                 405
AAGAGCATGGAGTACCGGTCCCGAGTGGTAGCGGACCCTCGTGGA
       420                 435                 450
GTGCTCAAGCGCGATTTCGGTTTCGACATCCCCGATGAGGTGGAG
```

```
                    465                 480                 495
GTCAGGGTTTGGGACAGCAGCTCCGAAATCCGCTACATCGTCATC
                    510                 525                 540
CCGGAACGGCCGGCCGGCACCGACGGTTGGTCCGAGGAGGAGCTG
                    555                 570                 585
ACGAAGCTGGTGAGCCGGGACTCGATGATCGGTGTCAGTAATGCG
                    600
CTCACACCGCAGGAAGTGATCGTA
```

(6) INFORMATION FOR SEQ ID NO: 6

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 687 nucleic acids

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\beta^{(H)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6

```
                    15                  30                  45
ATGGATGGTATCCACGACACAGGCGGCATGACCGGATACGGACCG
                    60                  75                  90
GTCCCCTATCAGAAGGACGAGCCCTTCTTCCACTACGAGTGGGAG
                    105                 120                 135
GGTCGGACCCTGTCAATTCTGACTTGGATGCATCTCAAGGGCATA
                    150                 165                 180
TCGTGGTGGGACAAGTCGCGGTTCTTCCGGGAGTCGATGGGGAAC
                    195                 210                 225
GAAAACTACGTCAACGAGATTCGCAACTCGTACTACACCCACTGG
                    240                 255                 270
CTGAGTGCGGCAGAACGTATCCTCGTCGCCGACAAGATCATCACC
```

```
                  285                 300                 315
GAAGAAGAGCGAAAGCACCGTGTGCAAGAGATCCTTGAGGGTCGG
                  330                 345                 360
TACACGGACAGGAAGCCGTCGCGGAAGTTCGATCCGGCCCAGATC
                  375                 390                 405
GAGAAGGCGATCGAACGGCTTCACGAGCCCCACTCCCTAGCGCTT
                  420                 435                 450
CCAGGAGCGGAGCCGAGTTTCTCTCTCGGTGACAAGATCAAAGTG
                  465                 480                 495
AAGAGTATGAACCCGCTGGGACACACACGGTGCCCGAAATATGTG
                  510                 525                 540
CGGAACAAGATCGGGGAAATCGTCGCCTACCACGGCTGCCAGATC
                  555                 570                 585
TATCCCGAGAGCAGCTCCGCCGGCCTCGGCGACGATCCTCGCCCG
                  600                 615                 630
CTCTACACGGTCGCGTTTTCCGCCCAGGAACTGTGGGGCGACGAC
                  645                 660                 675
GGAAACGGGAAAGACGTAGTGTGCGTCGATCTCTGGGAACCGTAC
CTGATCTCTGCG
```

(7) INFORMATION FOR SEQ ID NO: 7

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 621 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous J-1

(FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\alpha^{(L)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

```
                    15                  30                  45
ATGACCGCCCACAATCCCGTCCAGGGCACGTTGCCACGATCGAAC
                    60                  75                  90
GAGGAGATCGCCGCACGCGTGAAGGCCATGGAGGCCATCCTCGTC
                   105                 120                 135
GACAAGGGCCTGATCTCCACCGACGCCATCGACCACATGTCCTCG
                   150                 165                 180
GTCTACGAGAACGAGGTCGGTCCTCAACTCGGCGCCAAGATCGTC
                   195                 210                 225
GCCCGCGCCTGGGTCGATCCCGAGTTCAAGCAGCGCCTGCTCACC
                   240                 255                 270
GACGCCACCAGCGCCTGCCGTGAAATGGGCGTCGGCGGCATGCAG
                   285                 300                 315
GGCGAAGAAATGGTCGTGCTGGAAAACACCGGCACGGTCCACAAC
                   330                 345                 360
ATGGTCGTATGTACCTTGTGCTCGTGCTATCCGTGGCCGGTTCTC
                   375                 390                 405
GGCCTGCCACCCAACTGGTACAAGTACCCCGCCTACCGCGCCCGC
                   420                 435                 450
GCTGTCCGCGACCCCCGAGGTGTGCTGGCCGAATTCGGATATACC
                   465                 480                 495
CCCGACCCTGACGTCGAGATCCGGATATGGGACTCGAGTGCCGAA
                   510                 525                 540
CTTCGCTACTGGGTCCTGCCGCAACGCCCAGCCGGCACCGAGAAC
                   555                 570                 585
TTCACCGAAGAACAACTCGCCGACCTCGTCACCCGCGACTCGCTC
                   600                 615
ATCGGCGTATCCGTCCCCACCACACCCAGCAAGGCC
```

(8) INFORMATION FOR SEQ ID NO: 8

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 678 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\beta^{(L)}$-subunit

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

```
                15                  30                  45
ATGGATGGAATCCACGACCTCGGTGGCCGCGCCGGCCTGGGTCCG
            60                  75                  90
ATCAAGCCCGAATCCGATGAACCTGTTTTCCATTCCGATTGGGAG
        105                 120                 135
CGGTCGGTTTTGACGATGTTCCCGGCGATGGCGCTGGCCGGCGCG
    150                 165                 180
TTCAATCTCGACCAGTTCCGGGGCGCGATGGAGCAGATCCCCCCG
195                 210                 225
CACGACTACCTGACCTCGCAATACTACGAGCACTGGATGCACGCG
240                 255                 270
ATGATCCACCACGGCATCGAGGCGGGCATCTTCGATTCCGACGAA
285                 300                 315
CTCGACCGCCGCACCCAGTACTACATGGACCATCCGGACGACACG
330                 345                 360
ACCCCCACGCGGCAGGATCCGCAACTGGTGGAGACGATCTCGCAA
375                 390                 405
CTGATCACCCACGGAGCCGATTACCGACGCCCGACCGACACCGAG
420                 435                 450
GCCGCATTCGCCGTAGGCGACAAAGTCATCGTGCGGTCGGACGCC
465                 480                 495
TCACCGAACACCCACACCCGCCGCGCCGGATACGTCCGCGGTCGT
510                 525                 540
GTCGGCGAAGTCGTGGCGACCCACGGCGCGTATGTCTTTCCGGAC
555                 570                 585
ACCAACGCACTCGGCGCCGGCGAAAGCCCCGAACACCTGTACACC
600                 615                 630
GTGCGGTTCTCGGCGACCGAGTTGTGGGGTGAACCTGCCGCCCCG
645                 660                 675
AACGTCGTCAATCACATCGACGTGTTCGAACCGTATCTGCTACCG
GCC
```

(9) INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\alpha^{(H)}$-subunit: $\alpha_1^{(H)}$

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

Ser-Glu-His-Val-Asn-Lys-Tyr-Thr-Glu-Tyr-Glu-Ala-Arg-Thr-Lys
Ala-Ile-Glu-Thr-Leu-Leu-Tyr-Glu-Arg-Gly-Leu

(10) INFORMATION FOR SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\beta^{(H)}$-subunit: $\beta_1^{(H)}$

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

Met-Asp-Gly-Ile-His-Asp-Thr-Gly-Gly-Met-Thr-Gly-Tyr-Gly-Pro
Val-Pro-Tyr-Gln-Lys-Asp-Glu-Pro-Phe-Phe-His-Tyr-Glu

(11) INFORMATION FOR SEQ ID NO: 11

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\alpha^{(L)}$-subunit: $\alpha_1^{(L)}$

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

Thr-Ala-His-Asn-Pro-Val-Gln-Gly-Thr-Leu-Pro-Arg-?-Asn-Glu
5 10 15

(12) INFORMATION FOR SEQ ID NO: 12

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

(A) OTHER INFORMATION

$\beta^{(L)}$-subunit: $\beta_1^{(L)}$

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

Met-Asp-Gly-Ile-His-Asp-Leu-Gly-Gly-Arg-Ala-?-Leu-?-Pro
5 10 15

Ile-Lys-Pro-Glu

(13) INFORMATION FOR SEQ ID NO: 13

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2070 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus sp.

(B) STRAIN: N-774 (FERM BP-1936)

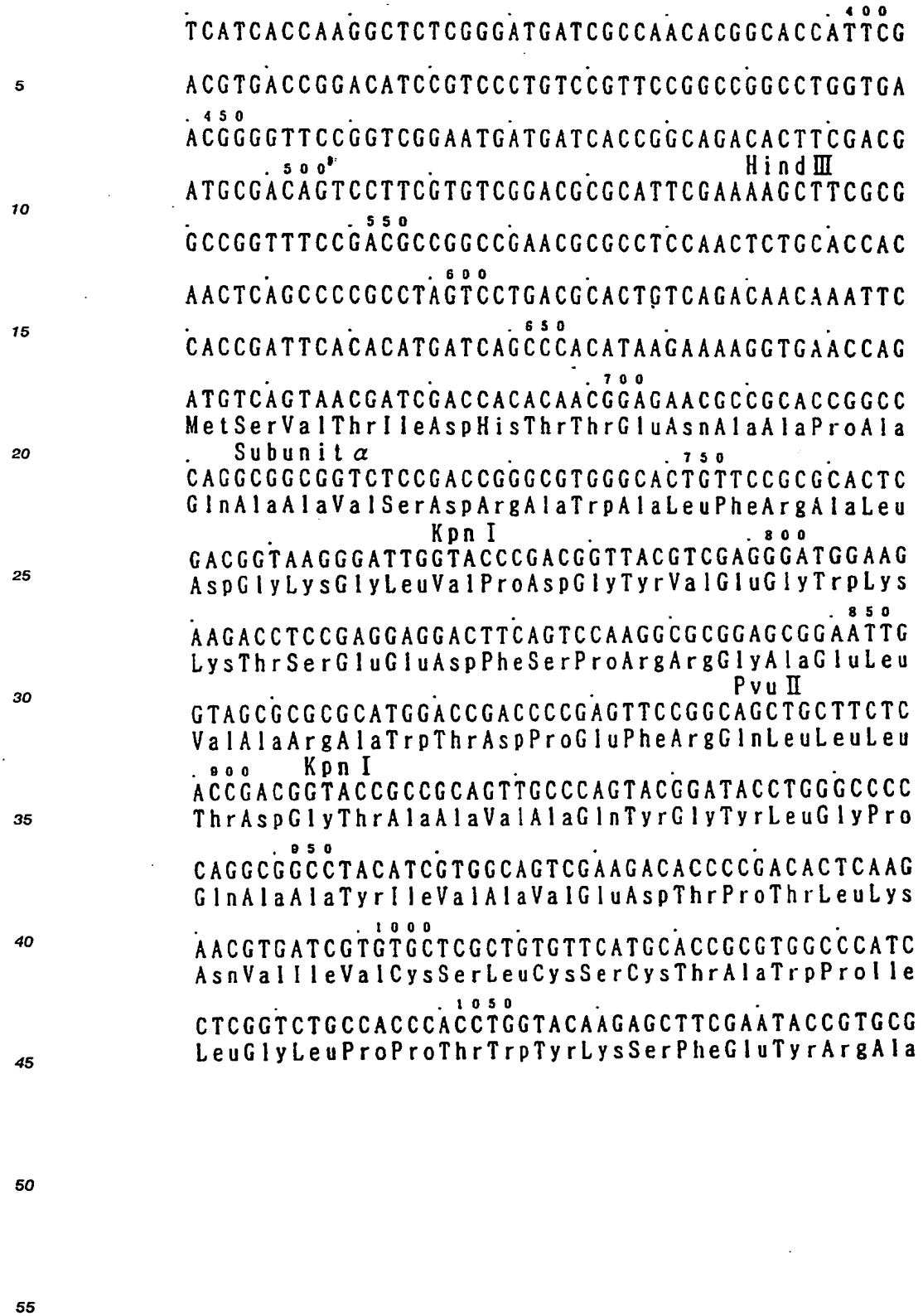
(ix) FEATURES

from nucleotide No. 675 to 1295: subunit α

from nucleotide No. 1225 to 1960: subunit β

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

SphI
GCATGCTTTCCACATCTGGAACGTGATCGCCACGGACGGTGGTG
CCTACCAGATGTTGGACGGCAACGGATACGGCATGAACGCCGAAG
GTTTGTACGATCCGGAACTGATGGCACACTTTGCTTCTCGACGCA
TTCAGCACGCCGACGCTCTGTCCGAAACCGTCAAACTGGTGGCCC
TGACCGGCCACCACGGCATCACCACCCTCGGCGGCGCGAGCTACG
GCAAAGCCCGGAACCTCGTACCGCTTGCCCGCGCCGCCTACGACA
CTGCCTTGAGACAATTCGACGTCCTGGTGATGCCAACGCTGCCCT
ACGTCGCATCCGAATTGCCGGCGAAGGACGTAGATCGTGCAACCT



```
                                        1100
CGCGTGGTCCGCGAACCACGGAAGGTTCTCTCCGAGATGGGAACC
ArgValValArgGluProArgLysValLeuSerGluMetGlyThr

                                    1150
GAGATCGCGTCGGACATCGAGATTCGCGTCTACGACACCACCGCC
GluIleAlaSerAspIleGluIleArgValTyrAspThrThrAla

                                              1200
GAAACTCGCTACATGGTCCTCCCGCAGCGTCCCGCCGGCACCGAA
GluThrArgTyrMetValLeuProGlnArgProAlaGlyThrGlu
                    PstI
                                         1250
GGCTGGAGCCAGGAACAACTGCAGGAAATCGTCACCAAGGACTGC
GlyTrpSerGlnGluGlnLeuGlnGluIleValThrLysAspCys

                                                   1300
CTGATCGGGGTTGCAATCCCGCAGGTTCCCACCGTCTGATCACCC
LeuIleGlyValAlaIleProGlnValProThrValTRM

CGACAAGAAGGAAGCACACC-ATGGATGGAGTACACGATCTTGCC
                     MetAspGlyValHisAspLeuAla
                     Subunit β

          1350
GGAGTACAAGGCTTCGGCAAAGTCCCGCATACCGTCAACGCCGAC
GlyValGlnGlyPheGlyLysValProHisThrValAsnAlaAsp

          1400
ATCGGCCCCACCTTTCACGCCGAATGGGAACACCTGCCCTACAGC
IleGlyProThrPheHisAlaGluTrpGluHisLeuProTyrSer
               1450                          SalI
CTGATGTTCGCCGGTGTCGCCGAACTCGGGGCCTTCAGCGTCGAC
LeuMetPheAlaGlyValAlaGluLeuGlyAlaPheSerValAsp

                    1500
GAAGTGCGATACGTCGTCGAGCGGATGGAGCCGGGCCACTACATG
GluValArgTyrValValGluArgMetGluProGlyHisTyrMet

                                   1550
ATGACCCCGTACTACGAGAGGTACGTCATCGGTGTCGCGACATTG
MetThrProTyrTyrGluArgTyrValIleGlyValAlaThrLeu

                                        1600
ATGGTCGAAAAGGGAATCCTGACGCAGGACGAACTCGAAAGCCTT
MetValGluLysGlyIleLeuThrGlnAspGluLeuGluSerLeu

                                              1650
GCGGGGGGACCGTTCCCACTGTCACGGCCCAGCGAATCCGAAGGG
AlaGlyGlyProPheProLeuSerArgProSerGluSerGluGly
```

```
                                                          1700
CGGCCGGCACCCGTCGAGACGACCACCTTCGAAGTCGGGCAGCGA
ArgProAlaProValGluThrThrThrPheGluValGlyGlnArg

                                                        1750
GTACGCGTACGCGACGAGTACGTTCCGGGGCATATTCGAATGCCT
ValArgValArgAspGluTyrValProGlyHisIleArgMetPro

GCATACTGCCGTGGACGAGTGGGAACCATCTCTCATCGAACTACC
AlaTyrCysArgGlyArgValGlyThrIleSerHisArgThrThr
 1800
GAGAAGTGGCCGTTTCCCGACGCAATCGGCCACGGGCGCAACGAC
GluLysTrpProPheProAspAlaIleGlyHisGlyArgAsnAsp
      1850
GCCGGCGAAGAACCGACGTACCACGTGAAGTTCGCCGCCGAGGAA
AlaGlyGluGluProThrTyrHisValLysPheAlaAlaGluGlu
          1900                                  SalI
TTGTTCGGTAGCGACACCGACGGTGGAAGCGTCGTTGTCGACCTC
LeuPheGlySerAspThrAspGlyGlySerValValValAspLeu

                1950
TTCGAGGGTTACCTCGAGCCTGCGGCCTGATCTTCCAGCATTCCA
PheGluGlyTyrLeuGluProAlaAlaTRM

                                  2000
GGCGGCGGTCACGCGATCACAGCGGTTCGTGCGACCGCCGCCTGA

                                        2050
TCACCACGATTCACTCATTCGGAAGGACACTGGAAATCATGGTCG
SalI
AC
```

(14) INFORMATION FOR SEQ ID NO: 14

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1970 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous J-1

(FERM BP-1478)

(ix) FEATURES

from nucleotide No. 408 to 1094: subunit $\beta^{(H)}$

from nucleotide No. 1111 to 1719: subunit $\alpha^{(H)}$

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

```
                10        20        30        40        50        60
CTGCAGCTCGAACATCGAAGGGTGCGAGCCGAGAGATCGGAGACGCAGACACCCGGAGGG

                70        80        90       100       110       120
AACTTAGCCTCCCGGACCGATGCGTGTCCTGGCAACGCCTCAAAATTCAGTGCAAGCGAT

               130       140       150       160       170       180
TCAATCTTGTTACTTCCAGAACCGAATCACGTCCCCGTAGTGTGCGGGGAGAGCGCCCGA

               190       200       210       220       230       240
ACGCAGGGATGGTATCCATGCGCCCCTTCTCTTTTCGAACGAGAACCGGCCGGTACAGCC

               250       260       270       280       290       300
GACCCGGAGACACTGTGACGCCGTTCAACGATTGTTGTGCTGTGAAGGATTCACCCAAGC

               310       320       330       340       350       360
CAACTGATATCGCCATTCCGTTGCCGGAACATTTGACACCTTCTCCCTACGAGTAGAAGC

               370       380       390       400       410       420
CAGCTGGACCCCTCTTTGAGCCCAGCTCCGATGAAAGGAATGAGGAAATGGATGGTATCC
                                                   MetAspGlyIleH
                                                   Subunit β (H)

               430       440       450       460       470       480
ACGACACAGGCGGCATGACCGGATACGGACCGGTCCCCTATCAGAAGGACGAGCCCTTCT
isAspThrGlyGlyMetThrGlyTyrGlyProValProTyrGlnLysAspGluProPheP

               490       500       510       520       530       540
TCCACTACGAGTGGGAGGGTCGGACCCTGTCAATTCTGACTTGGATGCATCTCAAGGGCA
heHisTyrGluTrpGluGlyArgThrLeuSerIleLeuThrTrpMetHisLeuLysGlyI

               550       560       570       580       590       600
TATCGTGGTGGGACAAGTCGCGGTTCTTCCGGGAGTCGATGGGGAACGAAAACTACGTCA
leSerTrpTrpAspLysSerArgPhePheArgGluSerMetGlyAsnGluAsnTyrValA

               610       620       630       640       650       660
ACGAGATTCGCAACTCGTACTACACCCACTGGCTGAGTGCGGCAGAACGTATCCTCGTCG
snGluIleArgAsnSerTyrTyrThrHisTrpLeuSerAlaAlaGluArgIleLeuValA

               670       680       690       700       710       720
CCGACAAGATCATCACCGAAGAAGAGCGAAAGCACCGTGTGCAAGAGATCCTTGAGGGTC
laAspLysIleIleThrGluGluGluArgLysHisArgValGlnGluIleLeuGluGlyA
```

```
          730         740         750         760         770         780
GGTACACGGACAGGAAGCCGTCGCGGAAGTTCGATCCGGCCCAGATCGAGAAGGCGATCG
rgTyrThrAspArgLysProSerArgLysPheAspProAlaGlnIleGluLysAlaIleG
          790         800         810         820         830         840
AACGGCTTCACGAGCCCCACTCCCTAGCGCTTCCAGGAGCGGAGCCGAGTTTCTCTCTCG
luArgLeuHisGluProHisSerLeuAlaLeuProGlyAlaGluProSerPheSerLeuG
          850         860         870         880         890         900
GTGACAAGATCAAAGTGAAGAGTATGAACCCGCTGGGACACACACGGTGCCCGAAATATG
lyAspLysIleLysValLysSerMetAsnProLeuGlyHisThrArgCysProLysTyrV
          910         920         930         940         950         960
TGCGGAACAAGATCGGGGAAATCGTCGCCTACCACGGCTGCCAGATCTATCCCGAGAGCA
alArgAsnLysIleGlyGluIleValAlaTyrHisGlyCysGlnIleTyrProGluSerS
          970         980         990        1000        1010        1020
GCTCCGCCGGCCTCGGCGACGATCCTCGCCCGCTCTACACGGTCGCGTTTTCCGCCCAGG
erSerAlaGlyLeuGlyAspAspProArgProLeuTyrThrValAlaPheSerAlaGlnG
         1030        1040        1050        1060        1070        1080
AACTGTGGGGCGACGACGGAAACGGGAAAGACGTAGTGTCGTCGATCTCTGGGAACCGT
luLeuTrpGlyAspAspGlyAsnGlyLysAspValValCysValAspLeuTrpGluProT
         1090        1100        1110        1120        1130        1140
ACCTGATCTCTGCGTGAAAGGAATACGATAGTGAGCGAGCACGTCAATAAGTACACGGAG
yrLeuIleSerAla                  MetSerGluHisValAsnLysTyrThrGlu
                                Subunit α (H)
         1150        1160        1170        1180        1190        1200
TACGAGGCACGTACCAAGGCGATCGAAACCTTGCTGTACGAGCGAGGGCTCATCACGCCC
TyrGluAlaArgThrLysAlaIleGluThrLeuLeuTyrGluArgGlyLeuIleThrPro
         1210        1220        1230        1240        1250        1260
GCCGCGGTCGACCGAGTCGTTTCGTACTACGAGAACGAGATCGGCCCGATGGGCGGTGCC
AlaAlaValAspArgValValSerTyrTyrGluAsnGluIleGlyProMetGlyGlyAla
         1270        1280        1290        1300        1310        1320
AAGGTCGTGGCCAAGTCCTGGGTGGACCCTGAGTACCGCAAGTGGCTCGAAGAGGACGCG
LysValValAlaLysSerTrpValAspProGluTyrArgLysTrpLeuGluGluAspAla
         1330        1340        1350        1360        1370        1380
ACGGCCGCGATGGCGTCATTGGGCTATGCCGGTGAGCAGGCACACCAAATTTCGGCGGTC
ThrAlaAlaMetAlaSerLeuGlyTyrAlaGlyGluGlnAlaHisGlnIleSerAlaVal
         1390        1400        1410        1420        1430        1440
TTCAACGACTCCCAAACGCATCACGTGGTGGTGTGCACTCTGTGTTCGTGCTATCCGTGG
PheAsnAspSerGlnThrHisHisValValValCysThrLeuCysSerCysTyrProTrp
```

```
          1450       1460       1470       1480       1490       1500
CCGGTGCTTGGTCTCCCGCCCGCCTGGTACAAGAGCATGGAGTACCGGTCCCGAGTGGTA
ProValLeuGlyLeuProProAlaTrpTyrLysSerMetGluTyrArgSerArgValVal
          1510       1520       1530       1540       1550       1560
GCGGACCCTCGTGGAGTGCTCAAGCGCGATTTCGGTTTCGACATCCCCGATGAGGTGGAG
AlaAspProArgGlyValLeuLysArgAspPheGlyPheAspIleProAspGluValGlu
          1570       1580       1590       1600       1610       1620
GTCAGGGTTTGGGACAGCAGCTCCGAAATCCGCTACATCGTCATCCCGGAACGGCCGGCC
ValArgValTrpAspSerSerSerGluIleArgTyrIleValIleProGluArgProAla
          1630       1640       1650       1660       1670       1680
GGCACCGACGGTTGGTCCGAGGAGGAGCTGACGAAGCTGGTGAGCCGGGACTCGATGATC
GlyThrAspGlyTrpSerGluGluGluLeuThrLysLeuValSerArgAspSerMetIle
          1690       1700       1710       1720       1730       1740
GGTGTCAGTAATGCGCTCACACCGCAGGAAGTGATCGTATGAGTGAAGACACACTCACTG
GlyValSerAsnAlaLeuThrProGlnGluValIleVal
          1750       1760       1770       1780       1790       1800
ATCGGCTCCCGGCGACTGGGACCGCCGCACCGCCCCGCGACAATGGCGAGCTTGTATTCA

          1810       1820       1830       1840       1850       1860
CCGAGCCTTGGGAAGCAACGGCATTCGGGGTCGCCATCGCGCTTTCGGATCAGAAGTCGT

          1870       1880       1890       1900       1910       1920
ACGAATGGGAGTTCTTCCGACAGCGTCTCATTCACTCCATCGCTGAGGCCAACGGTTGCG

          1930       1940       1950       1960       1970
AGGCATACTACGAGAGCTGGACAAAGGCGCTCGAGGCCAGCGTGGTCGAC
```

(15) INFORMATION FOR SEQ ID NO: 15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1731 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Rhodococcus rhodochrous

(B) STRAIN: J-1 (FERM BP-1478)

(ix) FEATURES

from nucleotide No. 171 to 848: subunit $\beta^{(L)}$

from nucleotide No. 915 to 1535: subunit $\alpha^{(L)}$

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15

```
                10        20        30        40        50        60
GAGCTCCCTGGAGCCACTCGCGCCGACGCATCCACGCTCGGACAGCCCACGGTGCGGATC

                70        80        90       100       110       120
ACCCCTGTTCGTCGGTAACAGAACAGTAACATGTCATCAGGTCATGACGTGTTGACGCAT

               130       140       150       160       170       180
TAGACGAGGGCACATAGGGTTGGTGACTCACGGCACAAGGAGAGCATTTCATGGATGGAA
                                                    MetAspGlyI
                                                    Subunit β (L)
               190       200       210       220       230       240
TCCACGACCTCGGTGGCCGCGCCGGCCTGGGTCCGATCAAGCCCGAATCCGATGAACCTG
leHisAspLeuGlyGlyArgAlaGlyLeuGlyProIleLysProGluSerAspGluProV
               250       260       270       280       290       300
TTTTCCATTCCGATTGGGAGCGGTCGGTTTTGACGATGTTCCCGGCGATGGCGCTGGCCG
alPheHisSerAspTrpGluArgSerValLeuThrMetPheProAlaMetAlaLeuAlaG
               310       320       330       340       350       360
GCGCGTTCAATCTCGACCAGTTCCGGGGCGCGATGGAGCAGATCCCCCCGCACGACTACC
lyAlaPheAsnLeuAspGlnPheArgGlyAlaMetGluGlnIleProProHisAspTyrL
               370       380       390       400       410       420
TGACCTCGCAATACTACGAGCACTGGATGCACGCGATGATCCACCACGGCATCGAGGCGG
euThrSerGlnTyrTyrGluHisTrpMetHisAlaMetIleHisHisGlyIleGluAlaG
               430       440       450       460       470       480
GCATCTTCGATTCCGACGAACTCGACCGCCGCACCCAGTACTACATGGACCATCCGGACG
lyIlePheAspSerAspGluLeuAspArgArgThrGlnTyrTyrMetAspHisProAspA
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          490        500        510        520        530        540
ACACGACCCCCACGCGGCAGGATCCGCAACTGGTGGAGACGATCTCGCAACTGATCACCC
spThrThrProThrArgGlnAspProGlnLeuValGluThrIleSerGlnLeuIleThrH
          550        560        570        580        590        600
ACGGAGCCGATTACCGACGCCCGACCGACACCGAGGCCGCATTCGCCGTAGGCGACAAAG
isGlyAlaAspTyrArgArgProThrAspThrGluAlaAlaPheAlaValGlyAspLysV
          610        620        630        640        650        660
TCATCGTGCGGTCGGACGCCTCACCGAACACCCACACCCGCCGCGCCGGATACGTCCGCG
alIleValArgSerAspAlaSerProAsnThrHisThrArgArgAlaGlyTyrValArgG
          670        680        690        700        710        720
GTCGTGTCGGCGAAGTCGTGGCGACCCACGGCGCGTATGTCTTTCCGGACACCAACGCAC
lyArgValGlyGluValValAlaThrHisGlyAlaTyrValPheProAspThrAsnAlaL
          730        740        750        760        770        780
TCGGCGCCGGCGAAAGCCCCGAACACCTGTACACCGTGCGGTTCTCGGCGACCGAGTTGT
euGlyAlaGlyGluSerProGluHisLeuTyrThrValArgPheSerAlaThrGluLeuT
          790        800        810        820        830        840
GGGGTGAACCTGCCGCCCCGAACGTCGTCAATCACATCGACGTGTTCGAACCGTATCTGC
rpGlyGluProAlaAlaProAsnValValAsnHisIleAspValPheGluProTyrLeuL
          850        860        870        880        890        900
TACCGGCCTGACCAGGTCATCCGGTCCACCCAGCGAGACGTCCCTTCACCACAGACAGAA
euProAla

          910        920        930        940        950        960
ACGAGCCCACCCCGATGACCGCCCACAATCCCGTCCAGGGCACGTTGCCACGATCGAACG
                MetThrAlaHisAsnProValGlnGlyThrLeuProArgSerAsnG
                Subunit α (L)

          970        980        990       1000       1010       1020
AGGAGATCGCCGCACGCGTGAAGGCCATGGAGGCCATCCTCGTCGACAAGGGCCTGATCT
luGluIleAlaAlaArgValLysAlaMetGluAlaIleLeuValAspLysGlyLeuIleS
         1030       1040       1050       1060       1070       1080
CCACCGACGCCATCGACCACATGTCCTCGGTCTACGAGAACGAGGTCGGTCCTCAACTCG
erThrAspAlaIleAspHisMetSerSerValTyrGluAsnGluValGlyProGlnLeuG
         1090       1100       1110       1120       1130       1140
GCGCCAAGATCGTCGCCCGCGCCTGGGTCGATCCCGAGTTCAAGCAGCGCCTGCTCACCG
lyAlaLysIleValAlaArgAlaTrpValAspProGluPheLysGlnArgLeuLeuThrA
         1150       1160       1170       1180       1190       1200
ACGCCACCAGCGCCTGCCGTGAAATGGGCGTCGGCGGCATGCAGGGCGAAGAAATGGTCG
spAlaThrSerAlaCysArgGluMetGlyValGlyGlyMetGlnGlyGluGluMetValV
         1210       1220       1230       1240       1250       1260
TGCTGGAAAACACCGGCACGGTCCACAACATGGTCGTATGTACCTTGTGCTCGTGCTATC
alLeuGluAsnThrGlyThrValHisAsnMetValValCysThrLeuCysSerCysTyrP
         1270       1280       1290       1300       1310       1320
CGTGGCCGGTTCTCGGCCTGCCACCCAACTGGTACAAGTACCCCGCCTACCGCGCCCGCG
roTrpProValLeuGlyLeuProProAsnTrpTyrLysTyrProAlaTyrArgAlaArgA
         1330       1340       1350       1360       1370       1380
CTGTCCGCGACCCCCGAGGTGTGCTGGCCGAATTCGGATATACCCCCGACCCTGACGTCG
laValArgAspProArgGlyValLeuAlaGluPheGlyTyrThrProAspProAspValG
```

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          1390        1400        1410        1420        1430        1440
AGATCCGGATATGGGACTCGAGTGCCGAACTTCGCTACTGGGTCCTGCCGCAACGCCCAG
IuIleArgIleTrpAspSerSerAlaGluLeuArgTyrTrpValLeuProGlnArgProA
          1450        1460        1470        1480        1490        1500
CCGGCACCGAGAACTTCACCGAAGAACAACTCGCCGACCTCGTCACCCGCGACTCGCTCA
laGlyThrGluAsnPheThrGluGluGlnLeuAlaAspLeuValThrArgAspSerLeuI
          1510        1520        1530        1540        1550        1560
TCGGCGTATCCGTCCCCACCACACCCAGCAAGGCCTGACATGCCCCGACTCAACGAACAA
leGlyValSerValProThrThrProSerLysAla
          1570        1580        1590        1600        1610        1620
CCCCACCCGGGTCTCGAAGCCAACCTCGGCGACCTGGTACAGAATCTGCCGTTCAACGAA

          1630        1640        1650        1660        1670        1680
CGAATCCCCCGCCGCTCCGGCGAGGTCGCCTTCGATCAGGCCTGGGAGATCCGCGCCTTC

          1690        1700        1710        1720        1730
AGCATTGCCACCGCATTGCATGGCCAGGGCCGATTCGAATGGGACGAATTC
```

Claims

1. A DNA$^{(H)}$ fragment encoding a polypeptide having nitrile hydratase activity, said polypeptide comprising the $\alpha^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 1 and the $\beta^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 2.

2. A DNA$^{(L)}$ fragment encoding a polypeptide having nitrile hydratase activity, said polypeptide comprising the $\alpha^{(L)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 3 and the $\beta^{(L)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 4.

3. The DNA$^{(H)}$ fragment of claim 1 which contains the nucleotide sequences of the $\alpha^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 5 and the $\beta^{(H)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 6.

4. The DNA$^{(L)}$ fragment of claim 2 which contains the nucleotide sequences of the $\alpha^{(L)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 7 and the $\beta^{(L)}$-subunit as defined in the Sequence Listing by SEQ ID: No. 8.

5. A recombinant DNA comprising a DNA$^{(H)}$ or DNA$^{(L)}$ of any one of claims 1-4 in a vector.

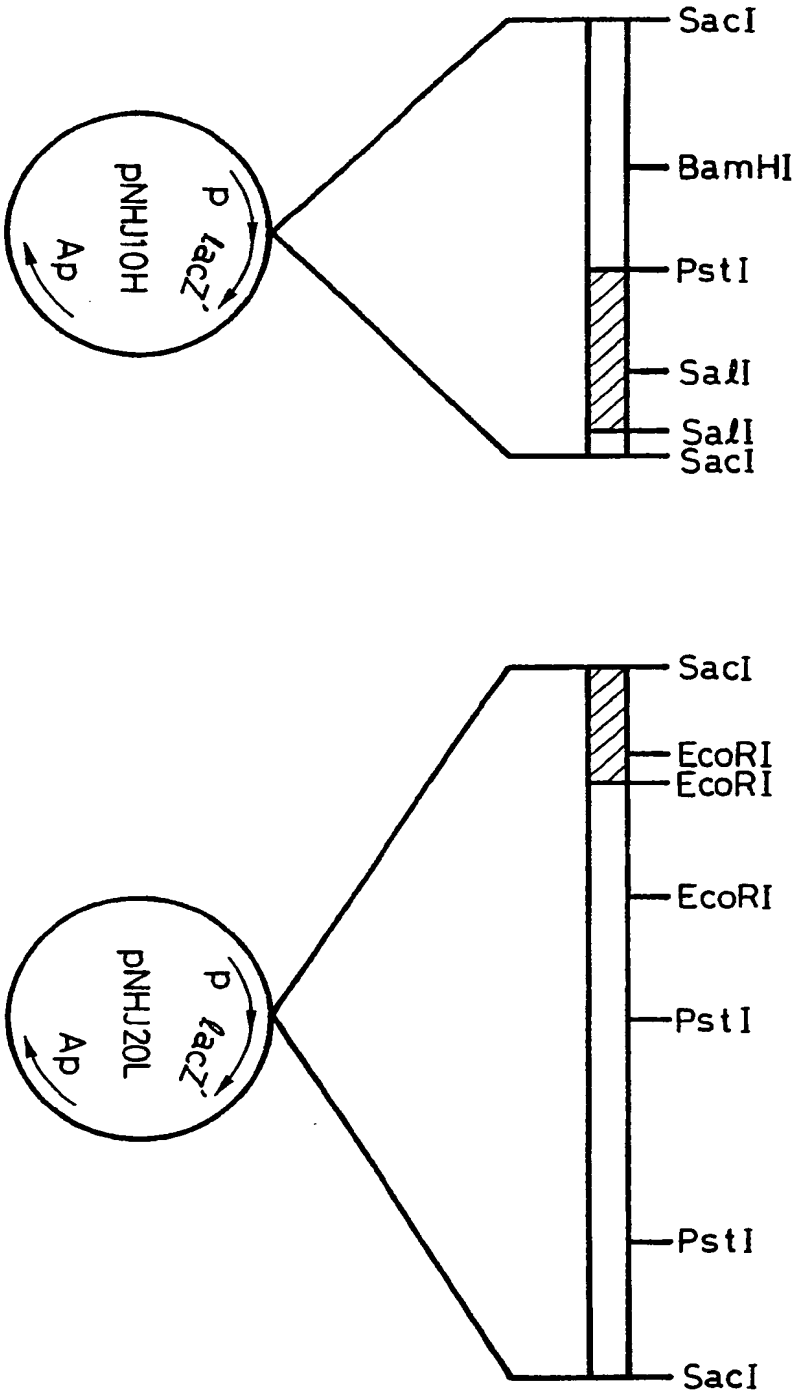
6. A transformant transformed with the recombinant DNA of claim 5.

7. A method of producing nitrile hydratase which comprises culturing the transformant as claimed in claim 6 and recovering nitrile hydratase from the culture.

8. A method of producing amides which comprises hydrating nitriles using nitrile hydratase obtained from the culture of the transformant of claim 6.

9. A method of producing amides which comprises culturing the transformant as claimed in claim 6, and hydrating nitriles to amides using the resultant culture, isolated bacterial cells, treated matter thereof, or a fixed material thereof.

FIG. 1


pNHJ10H
p
lacZ
Ap
SacI
BamHI
PstI
SalI
SalI
SacI
pNHJ20L
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lacZ
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SacI
EcoRI
EcoRI
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PstI
SacI

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(54) DNA fragment encoding a polypeptide having nitrile hydratase activity, a transformant containing the DNA fragment and a process for the production of amides using the transformant.

(57) The present invention discloses the amino acid sequence and nucleotide sequence of the α- and β-subunits of two types of nitrile hydratase derived from Rhodococcus rhodochrous J-I. The DNA fragment encoding nitrile hydratase is inserted into an expression vector and the recombinant vector is used for transformation. The transformant contains multiple copies of the gene and can produce much higher levels of nitrile hydratase than conventionally used microorganisms.

EP 0 445 646 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91102937.9

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,A	FR - A1 - 2 633 938 (TERUHIKO BEPPU et al.) * Claims * & JP-A-2-119 778 --	1,6-9	C 12 N 15/53 C 07 H 21/04 C 12 P 13/02
P,A	CHEMICAL ABSTRACTS, vol. 112, no. 13, March 26, 1990, Columbus, Ohio, USA O. IKEHATA et al. "Primary structure of nitrile hydratase deduced from the nucleotide sequence of a Rhodococcus species and its expression in Escherichia coli" page 176, right column, abstract-no. 112 972f & Eur. J. Biochem. 1989, 181 (3), 563-70 --	1	
D,A	EP - A2 - 0 307 926 (YAMADA et al.) * Abstract * ----	1,8,9	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N C 07 H C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	28-10-1991	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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